Functional mapping of the surface of *Escherichia coli* ribose-binding protein: Mutations that affect chemotaxis and transport

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Abstract

Ribose-binding protein is a bifunctional soluble receptor found in the periplasm of *Escherichia coli*. Interaction of liganded binding protein with the ribose high affinity transport complex results in the transfer of ribose across the cytoplasmic membrane. Alternatively, interaction of liganded binding protein with a chemotactic signal transducer, Trg, initiates taxis toward ribose. We have generated a functional map of the surface of ribose-binding protein by creating and analyzing directed mutations of exposed residues. Residues in an area on the cleft side of the molecule including both domains have effects on transport. A portion of the area involved in transport is also essential to chemotactic function. On the opposite face of the protein, mutations in residues near the hinge are shown to affect chemotaxis specifically.

Keywords: binding protein; chemotaxis; high affinity membrane transport; periplasm; protein-protein interactions; receptor; ribose; signal transduction

Gram-negative bacteria such as *Escherichia coli* use several different classes of transporters to transfer nutrients across the cytoplasmic membrane. One such class is that of the periplasmic binding protein-dependent transporters, also known as the osmotic shock-sensitive transport systems (Higgins et al., 1990). A wide variety of binding protein-dependent systems has been characterized, with specificities ranging from relatively large, uncharged maltodextrins to ions such as phosphate. Despite this diversity of substrates, all of these systems are organized similarly. Each of the binding protein-dependent systems uses a complex of proteins that traverses the inner membrane, serving to recognize liganded binding protein and to couple transport of the substrate against a concentration gradient to the hydrolysis of ATP. This membrane transport complex is composed of a hydrophilic protein, the putative ATPase, and one or more very hydrophobic proteins that serve to span the membrane (Higgins et al., 1990). Several mammalian homologs to the ATPase components have been discovered recently, including the cystic fibro-

sis-related protein and the multidrug resistance (P-glycoprotein) proteins (Hyde et al., 1990). In this study, we focus on the interaction between liganded binding protein and the membrane complex as a first step toward understanding the systems as a whole.

L-Arabinose (ABP)-, D-galactose (GBP)-, and D-ribose (RBP)-binding proteins serve as the initial receptors in the periplasm for high affinity membrane transport of their respective sugars in *E. coli* (Ames, 1986). GBP and RBP also function as the initial receptors for chemotaxis toward their respective sugars (Kondoh et al., 1979). Taxis toward arabinose does not occur. Although each binding protein interacts with its cognate membrane transport complex to transfer its sugar by active transport into the cell, GBP and RBP also interact with a common chemotaxis receptor, Trg, to initiate the chemotactic process (Kondoh et al., 1979; Bollinger et al., 1984).

The amino acid sequences (Hogg & Hermodson, 1977; Mahoney et al., 1981; Groarke et al., 1983) and the high resolution crystallographic structures (Quiocho & Vyas, 1984; Vyas et al., 1988; Mowbray et al., 1990; Mowbray & Cole, 1992) for ABP, GBP, and RBP have been determined. The primary structures are not very similar, but

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the tertiary structures are largely superimposable (Mowbray et al., 1990; Vyas et al., 1991; Mowbray, 1992), suggesting a highly conserved mechanism for transport amongst the three proteins. Each of the three proteins folds into two domains connected by a three-stranded hinge (Kinemage 1). As defined previously for RBP (Mowbray & Cole, 1992), Domain 1 contains the amino-terminus and consists largely of the first half of the linear sequence, and Domain 2 contains the carboxy-terminus and most of the second half of the sequence.

Because RBP and GBP compete for occupancy of Trg (Strange & Koshland, 1976), we and others (Argos et al., 1981; Park & Hazelbauer, 1986; Vyas et al., 1991) have considered the possibility that corresponding portions of the surfaces of these two binding proteins are similar enough to interact at a common binding site on Trg. The aligned sequences of ABP, GBP, and RBP were examined for segments where RBP and GBP are similar to each other, yet dissimilar to ABP, as judged by minimum base changes per codon criteria in 15-residue segments (Hermodson & Argos, unpubl.). A study of RBP structure and function was initiated employing site-directed mutagenesis of selected surface residues within the segments identified where the specific residues are very similar in GBP and RBP, with the aim of identifying the residues involved in chemotaxis. The phenotypes of the resulting mutant proteins allowed us to expand our goals to include determination of residues involved in transport.

This study begins to define the surfaces of RBP that are recognized by the ribose high affinity transport complex and by Trg. We demonstrate that the surface that mediates chemotaxis includes a portion of the surface involved in transport function.

Kehres and Hogg (1992 [companion paper]) have independently used a similar approach to map the sites on ABP that are involved in transport of arabinose. Although the studies were carried out independently, the data, when considered as a whole, suggest that these two proteins may share a subset of residues that is involved in a conserved transport mechanism.

Results

Mutational targeting and strategy

Analysis of the alignment of the amino acid sequences of ABP, GBP, and RBP identified six segments (RBP amino acids 38–41, 64–73, 90–100, 109–118, 129–138, and 223–231) in which the sequences of GBP and RBP were similar to each other, yet dissimilar from ABP. These segments were considered to be possible sites of chemotactic function because GBP and RBP share an interaction with Trg, whereas ABP does not. Initially the only binding protein coordinates available were those for ABP (Quiocho & Vyas, 1984), so the alignment was used to superimpose the selected segments of RBP sequence on the structure of ABP. Four of the segments (38–41, 64–73, 90–100,

129–138) comprise loops that form the lips of the substrate-binding cleft. Segment 109–118 is a helix on the opposite side of the molecule, which is adjacent to one of the three hinge strands (Kinemage 1), whereas segment 223–231 forms part of a helix and a loop near the C-terminus of the protein (far from the ligand-binding site). The prediction of the four areas near the lips of the cleft was in agreement with a previous model (Quiocho & Vyas, 1984) for the generation of a conformation capable of interaction with the transport complex via juxtaposition of residues in both domains of the protein. The prediction of segment 109–118, which occurs on the opposite face of the molecule, suggested an alternative model whereby the signal for chemotaxis was generated by a conformational change in the region near the hinge strands.

Because sites of protein–protein interaction must be found on the surface rather than the interior of a molecule, we chose to make substitutions at residues within the predicted chemotactic segments that were fully solvent exposed. Protein structures are likely to tolerate substitutions at such sites without gross structural changes, because exposed side chains are generally less important to the stability of the folded protein than are buried side chains. Although the segments predicted by sequence alignment proved useful for targeting the first few mutations, subsequent mutagenesis was guided more by the phenotypes of previous mutants. This approach allowed us to define areas involved in transport (for which we had no predictive algorithm) as well as that involved in chemotaxis. In general, we attempted to make conservative substitutions in areas with predicted function and radical substitutions near the edges of functional areas in order to define the extent of the interactive surfaces. Initially, all mutations were directed by nondegenerate oligonucleotides and produced defined substitutions at single sites. As the study progressed, degenerate oligonucleotides were designed to alter several contiguous surface residues in the same mutagenic reaction. The codon position(s) selected for degeneracy and the nucleotide mixtures included at these positions in the oligonucleotide syntheses were selected to produce the most informative amino acid substitutions at the sites in question. Although these oligonucleotides were biased to provide a preponderance of single-site point mutations, alleles with multiple substitutions were occasionally generated.

Characterization of mutant RBPs

The formation of a chemotactic ring on swarm plates (Adler, 1966) containing ribose as a sole carbon source is dependent upon the ability of RBP to interact productively with both the chemotactic transducer and the membrane-bound ribose transport proteins. Using ribose swarm plates as the initial test for mutations that affect either function, we have generated a large collection of

point mutations in RBP. Plasmids containing mutant RBP genes were used to transform the RBP deletion strain MH-6, and ribose swarm plate phenotypes were tested. Mutant alleles that reproducibly complemented strain MH-6 to form well-defined chemotactic rings whose rate of expansion was at least 80% of the rate of wild type were classified as silent mutations and not characterized further. This arbitrary cut-off limits detailed analysis to those mutations that either decrease transport by more than about 50% or reduce chemotactic response below an as yet ill-defined threshold. Mutant plasmids that failed to restore wild-type swarm plate phenotypes were sequenced between appropriate unique restriction sites, subcloned into wild-type vector, and retransformed into strain MH-6. The amount of periplasmic RBP present in plasmid-containing cells in stationary phase was estimated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) of proteins released by chloroform shock (data not shown). Several mutations (V36E/L37Q, D38A, D104R, A133D/G134A, D165R, D165V, D219V/ Δ K222/A223T, 264 frameshift) that caused a decrease in the level of RBP were presumed to alter synthesis, localization, folding, or stability and were not further characterized. Mutants that did not form normal tactic rings on

swarm plates but that did have normal levels of RBP in the periplasm were characterized in detail. Affinity for ribose was determined by equilibrium dialysis, chemotactic response was quantified by the capillary assay, and high affinity transport of ribose was measured by filtration.

Mutations that affect chemotaxis and/or transport

All mutant proteins in this section are properly localized in the cell, are present in concentrations similar to wild type, and retain K_d 's of $<1.0 \mu\text{M}$ for ribose (indicative of near-native tertiary structure). None of the mutations that allowed measurable transport caused a significant increase in the K_m for transport, but several showed a concurrent decrease in K_m and V_{max} . All of the mutant RBP-bearing cells exhibited peak accumulation in the capillary assay at either 10^{-3} or 10^{-4} M ribose and formed wild-type rings on galactose and tryptone swarm plates. The sites of the mutations are indicated in the crystallographic model in Figure 1 and Kinemage 2. References to secondary structural elements and domain locations in RBP are according to the designations shown on the schematic representation of the RBP structure (Fig. 2).

Fig. 1. Alpha-carbon and space-filling representations of ribose-binding protein mutations. Both are oriented identically; the view is directly into the sugar-binding cleft with the N-terminus-containing Domain 1 on the bottom and the C-terminus-containing Domain 2 on top. Sites of silent mutations are colored green, those that primarily affect transport are red, and those that affect both chemotaxis and transport are lavender. **A:** Stereo alpha-carbon tracing with untested residues drawn in white and mutations that primarily affect chemotaxis drawn in blue. **B:** Space-filling model with untested residues drawn in light blue. The mutations that primarily affect chemotaxis are occluded in this view. Models were produced with the program O (Jones et al., 1990).

The data for these mutant proteins are summarized in Table 1.

Class I: Severe effects on chemotaxis and transport

Four mutants were found to retain less than 20% peak chemotactic response and have a V_{max} for transport of less than 10% relative to wild type. All of the mutations that fall into this category are located quite close to the lips of the sugar-binding cleft and cluster on one face. A44E is the first fully solvent-exposed residue in helix B. A44E retains only 7% V_{max} for the transport function and 5% of the chemotactic function relative to wild type. On the adjacent helix C, also near the cleft, A70E retains no measurable transport and 16% of its chemotactic function. Somewhat farther away from the cleft, N73D reduces chemotaxis by 96% relative to wild type and exhibits transport that is near the lower limit of detection. Finally, across the cleft in Domain 2, G134 has been changed to R. This replacement abolishes both chemotaxis and transport.

Class II: Severe effects on transport but not chemotaxis

Seven mutations were found to reduce the V_{max} of transport by at least 49% without abolishing chemotaxis. These mutants were found to have mild chemotactic defects, retaining 30–70% peak ribose response relative to wild type. When L11 is replaced by either D or G, trans-

Table 1. Mutations that cause chemotactic and/or transport defects in ribose-binding proteins (RBPs)

RBP	K_d (μ M)	Relative ribose transport (mutant/wild type)		Relative peak chemotactic response (mutant/wild type)
		K_m	V_{max}	
Wild type	0.13	1.0	1.0	1.0
Class I. Loss of transport and chemotaxis				
A44E	0.21	0.29	0.07	0.05
A70E	0.15		<0.03 ^a	0.16
N73D	0.20		<0.03 ^a	0.04
G134R	0.04		<0.03 ^a	0.08
Class II. Loss of transport				
L11D	0.62	0.86	0.44	b
L11G	0.64	1.1	0.49	b
N12R	0.18	0.43	0.17	b
K45E	0.15	0.29	0.15	b
D67R	0.07		<0.03 ^a	b
G72D	0.10	0.57	0.44	b
D165A	0.37	1.1	0.51	b
Class III. Loss of chemotaxis				
L107R	0.33	1.3	0.78	0.30
I111R	0.86	1.1	1.1	0.38

^a Mutants in which ribose transport was less than 3% of the wild type at 10 μ M were tested at ribose concentrations up to 100 μ M, but K_m values could not be determined.

^b These mutants formed well-defined swarm rings on ribose swarm plates and had peak ribose capillary responses ranging from 30 to 70% of wild type. These relatively minor apparent chemotactic defects may be artifactual (see Discussion). Dissociation constants are the average of at least two trials where data form a straight-line Scatchard plot (Scatchard, 1949) with R^2 values >0.95. Reproducibility of these figures is about $\pm 10\%$. Transport K_m and V_{max} values are derived from Cornish-Bowden plots (Eisenthal & Cornish-Bowden, 1974) of data from at least two trials. Reproducibility for V_{max} and K_m is approximately $\pm 10\%$ and $\pm 25\%$, respectively. Chemotactic response is determined by normalizing the maximum number of cells that accumulate in capillary tubes in response to ribose to accumulation of the same cells responding to aspartate (Park & Hazelbauer, 1986). In all cases, the maximum accumulation occurred at 10^{-3} or 10^{-4} M ribose. Chemotactic responses reported are the average of at least two trials where accumulations varied no more than 15%. Wild-type transport $V_{max} = 59$ pmol 14 C-1-D-ribose per 10^6 cells per 25 s and $K_m = 0.7$ μ M. The peak accumulation of wild-type cells in the capillary assay occurred at 10^{-4} M ribose, with an average of 27,100 cells entering the capillary tube.

Fig. 2. Ribbon drawing of the structure of ribose-binding protein. Drawing produced by the graphics program Molscript (Kraulis, 1991).

port V_{max} is reduced by about 50%. This site is located between beta-strand 1 and helix A. N12R, in Domain 1 near the lip of the cleft, reduces transport V_{max} by about 85% relative to wild type. K45E, which also reduces transport V_{max} by about 85%, is located one turn down helix B from the binding cleft. D67R forms part of the lip of the binding cleft in Domain 1; it can be replaced by A, G, or V without phenotypic consequence, but R at this position specifically blocks transport function. G72D, the mutation analogous to a well-characterized G74D chemotaxis-specific lesion in GBP (Scholle et al., 1987; Vyas et al., 1988), has little effect on chemotaxis in RBP, but has a substantial effect on transport. D165A, located di-

rectly across the binding cleft from L11 and N12, also causes moderate (~50%) loss in transport while raising the K_d for ribose by about threefold.

Class III: Large effects on chemotaxis, but not transport

Only two mutations of this class were found. L107R and I111R, both located in helix D, reduce peak chemotaxis to 30–40% of wild type, whereas transport is relatively unaffected. The ribose dissociation constant of I111R is 0.86 μ M (6.6-fold higher than wild type), which suggests that the global conformation may be disturbed in such a way as to interfere with chemotactic signaling.

Silent mutations

The collection of silent mutations is summarized in Table 2 and depicted graphically in Figure 1 and Kimage 2.

At the start of the turn that connects beta-strand 1 to alpha-helix A, L11A and L11V are silent mutations, whereas L11G and L11D reduce transport.

In alpha-helix A, silent mutations have been isolated in various combinations at residues 17, 21, and 25. This zone begins near the lip of the sugar-binding cleft and extends away from the cleft.

Silent mutations Q40E, Q40A, and N41D are located in the turn connecting beta-strand 2 to helix B. The side chain of Q40 is completely solvent exposed, whereas the side chain of N41 extends over the binding cleft toward Domain 2. A44, near the N-terminus of helix B, can be replaced without functional effects by N or S, but A44E causes a loss of both transport and chemotaxis.

The turn between helix B and beta-strand 3 lies close to the N-terminus of RBP, on the face of Domain 1 farthest from the bound ribose. Mutation of K59 in this turn to E has no effect on swarm plate phenotype.

Residues 65–68 form the loop between beta-strand 3 and helix C. D67 is at the top of this loop, with the alpha-carbon located on the precipice of the cleft and the side chain pointing out from the cleft. Substitutions of A, G, or V are silent at D67, but D67R is not able to mediate transport.

D69 is on the lip of the cleft, extending toward Domain 2, and is in an alpha-turn preceding helix C. Substitution of D by G has no effect at this position. A70 points toward helix B and can be changed to G or V without affecting function, but E at this position reduces both transport and chemotaxis. Substitutions at positions 72 and 73 alter phenotype, but K76, whose side chain points extend out over the large loop between beta-strands 4 and 5, can be replaced by E without disturbing function. M77, which is oriented toward helix B, can be changed to E without consequence.

The central part of the loop connecting beta-strands 4 and 5 is composed of residues 93–96. Although proximate to residues 70–73 (in which mutations affect ring formation) substitutions at positions 93, 94, and 96 are all silent.

Helix D, a structural element in Domain 2, extends from residue 105 to residue 120 and is adjacent to one of the hinge strands. Substitutions I111A, D114N, D114R, and K118E have no effect, whereas L107R and I111R both show specific defects in chemotaxis.

K125 is the farthest residue from the binding pocket in beta-strand 6, and the side chain is fully exposed; K125E is a silent mutation.

The loop connecting beta-strand 6 to helix E composes part of the lip of the binding cleft in Domain 2. The substitution G134R affects both chemotaxis and transport, but either G or D can functionally replace A at position 133.

Helix E begins with the cleft residue 136 and ends far from the cleft at residue 152. Three mutations in this area, R139Q, E143A, and Q146K are all silent.

Residues 162–165 form the loop connecting beta-strand 7 to helix F and are part of the lip of the cleft. D165 points out away from the cleft and replacement with A causes a moderate defect in transport. D165G and D165G/I167S are both silent mutations.

Near the binding cleft in helix H, mutations D219A, D219A/K222E, and D219V/K222E are all silent.

Discussion

We have begun to define those surfaces of RBP that participate in protein–protein interactions by site-directed mutagenesis. The analysis was limited to those mutations causing an obvious phenotypic defect in chemotactic ring formation on ribose swarm plates. Because this assay is relatively insensitive, the actual interacting surfaces may extend beyond those described here.

This study has yielded considerable information regarding the surfaces involved in membrane transport. It was

Table 2. Silent mutations in ribose-binding proteins^a

L11A,V	K94E
V17D/D21K	E96Q
V17G/D21N	I111A
V17G/D21K	D114N,R
K25H	K118E
Q40A,E	K125E
N41D	A133D,G
A44N,S	R139Q
K59E	E143A
D67A,G,V	Q146K
D69G	D165G
A70G,V	D165G/I167S
K76E	D219A
M77E	D219A/K222E
T93A	D219V/K222E

^a A comma separates different substitutions at the same site. A slash denotes double mutations.

expected that residues on both sides of the binding protein cleft make close contact with the transport protein complex in order to ensure efficient transfer of the sugar. Recognition by the transporter (and/or the chemotactic transducer) of residues on both sides of the binding cleft, which are only adjacent when ligand is bound, provides a plausible mechanism for the discrimination of liganded versus nonliganded binding protein. This model is supported by our data. As seen in Figure 1 and Kinemage 2, all of the mutations that reduce transport are located close to the binding cleft.

On the face of RBP that includes the opening of the cleft, only three surface residues (K45, D67, and D165) are conserved in the structures of ABP, GBP, and RBP. Mutations at these positions in RBP indicate that each is important for transport function. Replacements at the analogous residues in ABP also reduce transport (Kehres & Hogg, 1992), suggesting that these residues participate in a common transport mechanism.

Detailed information regarding the surface characteristics required for transport has been obtained at several sites. At L11, replacements with A or V are silent, but replacements by G or D at this position interfere with transport, suggesting a requirement for a hydrophobic side chain. D67 can be functionally replaced by G or A, but transport is abolished by R, suggesting that the small size of D and not its carboxylate moiety is the most important factor. Although mutations near D67 in the tertiary structure (G134R and A70E) abolish chemotaxis, D67R retains more than half of wild-type chemotactic function. Amino acid 70, an A in the wild-type sequence, can be changed to G or V without effect, yet E at this site eliminates both chemotactic and transport function. This disruption might be caused by either the increased size of E or its negative charge.

Our data show that the surfaces involved in chemotactic and transport functions overlap extensively. In the current study, every mutation that causes defects in transport was also found to cause a reduction in chemotactic function. Several mutations that affect transport more than chemotaxis were isolated, however. Only two mutations were found that interfere with chemotaxis without affecting transport. This result is consistent with an earlier analysis of random mutations generated in GBP (Ordal & Adler, 1974). They also found only mutant GBPs which were defective in both chemotaxis and transport with one exception: G74D. Analysis of a large number of mutants in the maltose-binding protein, however, has demonstrated a spatial separation of the two functions (Spurlino et al., 1991; Zhang et al., 1992). This discrepancy may be related to the larger size of MBP or its more distant evolutionary relationship to RBP and GBP.

In spite of the overlap of functional sites, the algorithm that we used to target our initial mutations was successful in predicting areas involved in chemotaxis. Indeed, the entire surface defined by mutation to be involved in che-

motaxis is very similar in RBP and GBP, whereas mutations in nonconserved areas tend to have no effect on chemotaxis (e.g., substitutions at residues 139, 143, 146). The chemotactic surface is made up of the loop connecting beta-strand 6 and alpha-helix E near the sugar-binding cleft and N-terminal portions of helices B and C. The area surrounding G72 is of special interest because it corresponds to G74 in GBP, where the substitution G74D abolishes chemotaxis without affecting transport. The analogous mutation in RBP, however, has little effect on chemotaxis and reduces transport considerably. This surprising result can be explained by differences in the structures of GBP and RBP in this area. Examination of the crystallographic structures (Mowbray, 1992) reveals that these glycine residues, although they are in analogous positions, are oriented quite differently in space. The adjacent structure (to the right of G72 in Fig. 1) is also completely different in the two structures, with more space to accommodate a larger residue available in GBP than RBP (Mowbray, 1992). Thus, the introduction of D at G72 in RBP probably causes a subtle shift in helix C and/or a rearrangement of the neighboring structure to alleviate steric strain. The relatively conservative substitution N73D at the next position, which is totally solvent exposed, causes a severe chemotactic defect. Thus the same general area as that of GBP, though not the identical residues, seems to be involved in the interaction of RBP with Trg.

Although our data are consistent with a model in which RBP and GBP use similar surfaces to interact at one site on Trg, mutagenesis alone cannot provide proof. An analysis of Trg mutants identified two substitutions (R85H and G151D) that affect taxis to ribose and galactose differently (Park & Hazelbauer, 1986), which suggests that GBP and RBP may interact at distinct sites on the transducer. The recently published structure of the periplasmic domain of the related transducer Tar (Milburn et al., 1991) is of interest in this regard. Trg and Tar are expected to fold similarly in spite of rather dissimilar amino acid sequences (Moe & Koshland, 1986). Alignment of the sequences (data not shown) reveals that the Trg mutations are predicted to lie close to each other in a pair of loops on the end of the transducer farthest from the membrane. Moreover, the same area in Tar has been implicated for direct interaction with MBP by allele-specific suppressor mutations (Kossmann et al., 1988; Gardina et al., 1992). Thus, it is likely that GBP and RBP share the same site on Trg, although the details of the interaction may not be identical.

Two mutations, L107R and I111R, primarily affect chemotaxis and map on an entirely different face of the molecule from the other chemotactic mutations. Both are in helix D, the N-terminus of which is connected to one of the hinge strands (Kinemage 2). Because of this relationship to the hinge, we had postulated that this helix might change conformation upon ligand binding and therefore might be used to communicate the ribose-bound

conformation to Trg. Both L107R and I111R cause a loss in affinity for ribose, 2.5- and 6.6-fold, respectively, indicating that some aspect of the native structure has been compromised. Radical mutations farther from the hinge on the same helix (D114R and K118E), as well as the more conservative replacement I111A, do not cause major chemotactic defects. Although it is possible that both faces of RBP contact a Trg dimer or form RBP-RBP contacts during the signaling process, it is more likely that these mutations interfere with signaling by influencing the relative positions of the residues on the cleft side of the molecule via perturbation of hinge structure.

RBP has proven to be rather tolerant of substitution of surface residues. Although not discussed in detail here, in the few cases where mutations caused a decrease in levels of properly localized RBP, the effects are explainable in terms of structural stability. For instance, in the case of D38A, the possibility of forming a stabilizing H-bond to the main-chain nitrogen of residue 40 is compromised. The structural integrity of the mutant proteins was also assessed by measuring ribose dissociation constants. Because RBP is a very compact protein (Mowbray & Cole, 1992), even minor changes in tertiary structure are likely to perturb binding site geometry or hinge flexibility and thus alter K_d .

We have noticed that the transport-defective mutant proteins, although capable of producing well-defined swarm rings, seem to have substantial (30–70%) defects in chemotaxis when assayed by the capillary method. Assays of strains containing appropriate levels of RBP but lacking the genes for the membrane transport complex suggest that defects in transport may interfere with capillary assays for ribose chemotaxis (unpubl.). Because of this uncertainty, in this study we have only considered those mutants that do not form well-defined chemotactic rings on ribose swarm plates to have genuine chemotactic defects.

Materials and methods

Bacterial strains

Strain OW1 (Ordal & Adler, 1974) was obtained from G.L. Hazelbauer. The *recBC*, *sbcB* strain, JC7623, was obtained from R.L. Somerville. Strain BL21(λ DE3) was obtained from J.E. Dixon. Strain TG1 was supplied by Amersham as part of the site-directed mutagenesis kit. Plasmid pBCK is a pUC19-based plasmid that contains *rbs* operon sequences in which the segment from the *AccI* site near the 3' end of *rbsC* to the *NaeI* site in *rbsK* is replaced by the 3.8-kb *BamHI* (kanamycin resistance/sucrose sensitivity) cassette from plasmid pUM24 (Ried & Collmer, 1987). Plasmid pBCK was linearized and used to transform strain JC7623, and a kanamycin-resistant, sucrose-sensitive colony was selected. The resultant *rbsB*[−], *rbsK*[−] strain, JCK, was presumably formed by the re-

placement of the *rbsBK* genes by the drug-resistance/sucrose-sensitivity cassette via homologous recombination. P1 phage grown on strain JCK was used to transduce strain OW1, and the desired kanamycin-resistant, sucrose-sensitive, *rbs*-deleted strain was isolated and named OWK. Plasmid pAO6, carrying *rbsDACKR* (the entire *rbs* operon except for a segment beginning just after the stop codon for *rbsC* and ending at the *HincII* site in *rbsB*) was linearized and used to transform strain JCK, followed by selection for sucrose tolerance. A colony that was sucrose tolerant, kanamycin sensitive, and *rbsK*⁺ was selected and named JC-6. P1 grown on strain JC-6 was used to transduce strain OWK to sucrose tolerance, kanamycin sensitivity, and *rbsK*⁺. The resultant strain was named MH-6. Thus, strain MH-6 has all the *rbs* genes except for that encoding RBP. The absence of *rbsB* in MH-6 was verified by use of antiRBP antibodies and the inability to form a ring on ribose swarm plates (data not shown).

Cloning vector

RBP mutations were created and characterized using derivatives of phagemid p4B1. Phagemid p4B1 was designed to streamline the analysis of *rbsB* mutants by minimizing DNA sequencing and subcloning. In addition to the pMB1 origin from pBR322, this phagemid has an f1 origin which allows the production of single-stranded DNA (for sequencing and mutagenesis) when host cells are infected by helper phage M13K07 (Viera & Messing, 1987). The phagemid also has two promoters (one from phage T7 and one from pBR322) that permit the use of a single vector for overproduction and phenotypic characterization and thus eliminate the need to subclone mutants into a separate overexpression vector. The strong T7 promoter is very poorly recognized by *E. coli* RNA polymerase and demonstrates negligible activity in wild-type strains. To overexpress mutant proteins for purification and characterization, phagemids were transformed into strain BL21(λ DE3) (Studier & Moffatt, 1986), and the cells were induced with isopropyl- β -D-thiogalactopyranoside (IPTG). In this strain, expression of T7 RNA polymerase is driven by the IPTG-inducible *lac* promoter. Synthesis of the T7 polymerase causes extreme overexpression of genes coupled to the T7 promoter, in this case the mutant RBP. The second promoter (originally located upstream of the promoter for the tetracycline resistance gene in pBR322) provides a moderate, constitutive level of RBP expression. Control experiments demonstrated that chemotaxis and transport are similar in strain MH-6 harboring plasmid p4B1 and the wild-type parent OW1, which contains a single chromosomal copy of the *rbsB* gene (data not shown). The steps involved in construction of p4B1 are detailed below.

Plasmid pBR322 was digested with *HindIII* and *PvuII*, and the 2.3-kb fragment was isolated. Phage pSBT3mp10

(M13 with *rbsB*) was digested with HindIII and NaeI and the 1.4-kb fragment was isolated. The above two fragments were ligated, producing plasmid pHZB1. Plasmid pHZB1 was digested with ScaI and SspI, and the 3.4-kb fragment was isolated. Phagemid pBluescriptIIS/K(–) was digested with ScaI and HincII, and the 1.1-kb fragment was isolated. These two fragments were ligated to form phagemid pT7B1(–). Phagemid pT7B1(–) was digested with XmnI and KpnI, and the 3.5-kb fragment was isolated. Phagemid pBluescriptIIS/K(+) was digested with the same enzymes, and the 1.1-kb fragment was isolated. Ligation of the two fragments yielded phagemid, pT7B1(+). Four silent mutations creating unique restriction sites (SacII at amino acid 55, XbaI at amino acid 88, SmaI at amino acid 139, and ApaI at amino acid 197) were introduced consecutively to the *rbsB* coding sequence in pT7B1(+) by site-directed mutagenesis. The phagemid with all four engineered sites was named p4B1.

Overproduction and partial purification of mutant proteins

Phagemids bearing mutant *rbsB* genes were transformed into strain BL21(ΔDE3). Cultures were grown overnight at 37 °C with aeration in LB + 100 μg/mL ampicillin. Overnight cultures were diluted 1:200 into fresh LB + ampicillin and grown at 37 °C with aeration. When cultures reached an optical density (OD)₅₉₀ of approximately 1.0, T7 transcription was induced by adding IPTG to a final concentration of 0.1 mM. Cells were harvested after 8–12 h. Periplasmic proteins were prepared by a large-scale modification of the chloroform shock method of Ames (Ames et al., 1984). Induced cells were harvested by centrifugation in 500-mL bottles for 10 min at 8,000 × *g*. Cell pellets were resuspended in residual medium, and 10 mL of chloroform was added for each liter of culture. The suspension was mixed and allowed to sit for 15 min at room temperature. Fifty milliliters of 10 mM Tris (pH 8.0) was added per liter of culture, and the mixture was centrifuged for 10 min at 10,000 × *g*. The upper phase of this extraction was harvested, ammonium sulfate was added to 40% saturation, and the solution was centrifuged for 20 min at 10,000 × *g*. The supernatant containing binding protein was saturated with ammonium sulfate and centrifuged for 20 min at 10,000 × *g*. The resulting pellet was resuspended in about 10 mL of 10 mM Tris (pH 8.0) and dialyzed extensively against the same buffer at 4 °C. The resultant protein solutions were greater than 80% pure RBP as estimated by Coomassie blue-stained SDS-PAGE.

Determination of *K_d*

K_d values for wild-type and mutant proteins were determined by equilibrium dialysis using 0.25-mL dialysis chambers and membranes (molecular weight cut-off

10–12 kDa) from Hoefer Scientific Instruments. Partially purified RBP (0.25 mL) was dialyzed against 0.25 mL ¹⁴C-1-D-ribose (Amersham) in 10 mM Tris (pH 8.0). The concentration of RBP was approximately 0.5 μM, and ribose concentrations were varied from 0.02 μM to 10 μM. Radioactivity from 0.20-mL aliquots from each side of the chambers was counted.

Chemotaxis assays

All procedures for measuring chemotaxis are based on those of Adler as modified by Hazelbauer (Park & Hazelbauer, 1986; Hazelbauer, pers. comm.). Cells used in chemotaxis assays were selected for motility by successively picking cells from the edge of swarm rings on two tryptone swarm plates followed by one minimal maltose swarm plate.

Ribose swarm plates were inoculated with motile cells from tryptone broth, incubated in a humid incubator at 35 °C and scored after 18–24 h. For capillary assays, fully motile cells were grown to midlog-phase in tryptone broth + 100 μg/mL ampicillin, then diluted into H1 salts + 100 μg/mL ampicillin + 0.4% glycerin and 0.2% ribose. Cells were grown until two to three doublings had occurred, washed three times, and suspended in chemotaxis buffer (0.1 mM EDTA, 10 mM potassium phosphate, pH 7.0) at 1–2 × 10⁶ cells/mL. One-microliter micropipettes, sealed at one end and containing various concentrations of attractant, were inserted into 0.4 mL of the cell suspension in chambers formed by a bent capillary covered by a coverslip at 30 °C. After 45 min, micropipettes were removed, emptied, and aliquots were plated to determine the number of cells that had entered the tube. Chemotactic response to ribose was normalized by comparison to response of the same cells to aspartate.

Media

Luria broth, tryptone broth, and M9 minimal salts were prepared as described by Miller (1972). M9 and H1 (Hazelbauer et al., 1969) minimal salts were supplemented with threonine, histidine, and leucine at 0.25 g/L and thiamine at 10 μM. All media contained ampicillin at 100 μg/mL. Swarm plates contained 0.25% agar (Bacto) and either 1% tryptone and 0.5% NaCl or H1 minimal salts plus required amino acids, thiamine, and 0.1 mM ribose or maltose.

Transport assays

Transport assays were performed essentially as described by Hazelbauer (Iida et al., 1984), but with minor modifications. Strains were grown overnight in tryptone broth at 35 °C with shaking. Overnight cultures (400 μL) were diluted into 10 mL M9 + 0.4% glycerin + 0.2% ribose and grown to an OD₅₉₀ of ~0.8. Cells were washed and

pelleted three times in ice-cold 10 mM KHPO₄ (pH 7.0), 40 µg/mL chloramphenicol, and resuspended at OD₅₉₀ = 0.03 in 100 mM KHPO₄. Cells were warmed for 10 min at 30 °C, then 990 µL of cell suspension was added to tubes containing 110 µL ¹⁴C-ribose in the same buffer (final concentrations of 0.1 µM, 0.5 µM, 1.0 µM, 2.0 µM, and 3.0 µM). After 25 s, 1 mL of cell suspension was filtered and washed with 5 mL ice-cold wash buffer (10 mM Tris, pH 7.0, 0.5 mM MgCl₂, and 0.15 M NaCl). Filters were dried and radioactivity quantified using "Ready Protein Plus" scintillation fluid (Beckman).

Site-directed mutagenesis

Mutagenesis was performed according to the protocols provided with the Amersham "Oligonucleotide-directed in vitro mutagenesis system, Version 2," except that annealing was accomplished by heating the template and primer to 90 °C in a heating block for 3 min, then removing the block from the heater and allowing the mixture to cool gradually to below 30 °C.

Protein sequence

The amino acid sequence of RBP and the nucleotide sequence of the *rbs* operon are included in Genbank, accession number M13169. The crystallographic coordinates for the RBP structure have been submitted to the Brookhaven Protein Data Bank and assigned the reference number 1DRI (scheduled for release in September 1992).

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